Intracellular signalling: **Is PIP_2 a messenger too?**

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The phospholipid phosphatidylinositol (4,5) bisphosphate (PIP_2) has recently been shown to act downstream of the small G proteins Rac and Arf. Different effectors may be employed in each case, suggesting that PIP_2 has multiple signalling roles.

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In addition to its well-publicised role as precursor of the second messengers inositol (1,4,5) trisphosphate (IP_3), diacylglycerol and phosphatidylinositol (3,4,5) trisphosphate (PIP_3), phosphatidylinositol (4,5) bisphosphate (PIP_2) is involved in a variety of other processes, regulating actin-binding proteins, influencing protein localisation by binding to pleckstrin homology (PH) domains, and participating in intracellular membrane transport at a number of levels [1]. Given its diverse range of potential functions, it is clearly necessary for there to be some kind of functional compart-

The enzymes responsible for bulk PIP_2 synthesis are the type I PIP kinases (PIPkins), of which three isoforms — α, β and γ — have been cloned. Until recently, the mechanisms regulating these enzymes were obscure, but recent work has begun to dissect out a function for at least one of them, the type Iα PIPkin, in relaying signals from small G proteins. Although the idea of G-protein-regulated PIP_2 synthesis is not new, definitive identification of the G proteins — and indeed of the PIPkins — involved was previously lacking. Recent developments, however, provide compelling evidence for functional interactions between type I PIPkins and Rac-1 [2,3], Arf-6 [4] and, possibly, other members of the Arf family [4–6].

PIPkin regulation by Rac-1

The small G protein Rac-1 has an important role in the regulation of the actin cytoskeleton, promoting the formation of membrane ruffles (lamellipodia). Although Rac-1 has multiple effectors, its carboxyl terminus associates physically with both type Iα and type Iβ PIPkins, suggesting that one of its functions is to regulate PIP_2 production [3]. The associated PIPkin activity is the same irrespective of whether or not Rac-1 is in its active, GTP-bound form. Rather than stimulating PIPkin's catalytic activity, therefore, the G protein's role may be to recruit the PIPkin to its site of action.

The functional importance of this G protein–PIPkin association is demonstrated by the fact that it is essential for Rac-1-stimulated actin polymerisation in permeabilised platelets. The stimulation by Rac-1 of actin filament uncapping — a necessary prerequisite of actin polymerisation — requires its association with a catalytically active PIPkin, and is abolished by PIP_2-binding peptides, suggesting a direct role for PIP_2 in the uncapping process [2]. As Tolias *et al.* [3] report elsewhere in this issue, the PIPkin isoform involved in the function of Rac-1 in platelets has now been identified as type Iα. Intriguingly, although the type Iβ isoform does not affect actin polymerisation in this system, it *does* bind to Rac-1, and may therefore mediate some of the effects of the G protein on actin polymerisation (or other processes) in other tissues.

Arf-stimulated PIPkin activity

Small G proteins of the Arf family are involved in several distinct membrane transport pathways, and are activators of phospholipases D (PLDs). Of the six known mammalian isoforms, the best characterised, Arf-1, is involved in Golgi function. Several studies have shown that Arfs activate a type I-like PIPkin activity in membrane preparations [5,6], acid (PA), the product of PLD activity, to stimulate type I PIPkins has led to the suggestion that Arf influences PIPkin activity indirectly, by increasing PA production [5]. In agreement with this idea, a recent study [8] has shown that PLD-derived PA does indeed stimulate PIP_2 production during clathrin coat assembly on lysosomes *in vitro*.

In none of these cases has the PIPkin isoform involved been identified unequivocally, and in most cases a mixture of Arfs was used. But a defined Arf isoform, Arf-6, has now been shown to regulate a particular PIPkin, type Iα [4]: a G protein preparation from bovine brain that activates this PIPkin was identified as a mixture of Arfs, and although GTP-loaded Arf-1, Arf-5 and Arf-6 all activated the PIPkin *in vitro*, only Arf-6 co-localised with it in transfected cells. Moreover, only activated Arf-6 recruited the PIPkin to a defined location, lamellipodia. The observed activation of the PIPkin by Arfs was not, in this case, due to an indirect activation via PA. Instead, GTP-bound Arf activated PIP_2 synthesis only in the presence of exogenous PA. This apparent contradiction of the earlier study [8] may arise from the different experimental methods used: the more recent study [4] used purified proteins, whereas in the other [8], lysosomal preparations were employed. Given this evidence, it is possible that Arfs are capable of dual regulation of PIPkins *in vivo*: indirectly, via enhanced production of

the allosteric activator, PA, and by direct interaction with the PA-bound PIPkin. Together, these two mechanisms may result in synergistic PIPkin activation [4].

Although only Arf-6 co-localises with overexpressed type Iα PIPkin, other Arfs may also regulate distinct PIPkin isoforms. The Arf-regulated PIPkins in membrane preparations have not yet been identified [5,6,8], and it will be interesting to discover which isoform is involved: the idea that distinct type I PIPkins could act downstream of different G proteins is an attractive one. Given its stimulation by Arf-1 and Arf-5, type Iα PIPkin could even be involved, although its failure to co-localise with either of these proteins argues against this idea [4].

The functional consequences of Arf-mediated PIPkin activation at different locations are also likely to differ. Whereas Arf-6-mediated PIPkin activation at the plasma membrane is involved in membrane ruffling [4], and Arf-mediated PIPkin recruitment to the Golgi may be involved in vesicle budding [7], Arf-mediated PIP_2 synthesis is needed for secretion in permeabilised HL-60 cells [5]. Again, the Arf isoform has not been unequivocally identified, the PIPkin isoform is unknown, and the role played by the PIP_2 itself is unclear.

Consequences of G-protein-stimulated PIP_2 production

The function of the PIP_2 synthesised in response to small G protein signals is becoming clearer in some instances. It is apparent that the PIPkin bound to Rac-1 is needed for the PIP_2 -dependent uncapping of actin filaments prior to actin polymerisation [2,3]. In other cases, however, the PIP_2 produced may have distinct functions. Both mammalian PLDs, PLD-1 and PLD-2, require PIP_2 as an essential co-factor for activity, leading to the suggestion that a positive feedback loop exists in vesicle bud formation in membrane transport, such that PLD-derived PA stimulates PIP_2 production, in turn leading to enhanced PA synthesis [9]. Moreover, Hodgkin *et al.* [10] have now identified the PIP_2-binding site on PLD-1, a PH domain. Mutations preventing PIP_2 binding affect the activity of PLD-1, and deletion of the PH domain alters the protein's subcellular distribution (it is normally restricted to the Golgi/endosomal compartment), suggesting that PIP_2 synthesis may be essential for recruitment of PLD-1 to the correct site and for its activity. It remains to be seen whether the Arf-6-mediated increases in PIP_2 concentration in membrane ruffles are responsible for the observed recruitment of PLD-2 to these structures [4].

How can we begin to make sense of these various strands of information? The role of PIP_2 production in mediating the effects of small G proteins is a complex affair, which we are some way from fully understanding. But some common threads are beginning to emerge. It is interesting to note that, not only do both Rac-1 and Arf-6 interact with the same PIPkin isoform, type Iα, but also that both are implicated in membrane 'ruffling'. Arf-6 appears to act downstream of Rac-1 in this process, and it is tempting to speculate that these G proteins cooperate in producing the localised PIP_2 increase needed for actin filament uncapping as a component of the ruffling mechanism. Rac-1 may recruit the PIPkin to the membrane, where other regulatory factors, perhaps Arf-6 itself (in combination with PA), activate it. Together with other, PIP_2-independent processes also set in motion by these G proteins, this regulated PIP_2 production will ultimately result in lamellipodia formation. The role of Arf-mediated PIPkin regulation in Golgi function and secretion is more obscure, largely because of our ignorance of the real identities of the proteins involved, but again it seems that recruitment of a cytosolic PIPkin to its site of action is an important initial step.

Whatever the actual mechanisms underlying these stimulated increases in PIP_2 synthesis turn out to be, one thing seems clear: cells possess multiple pathways for the localised regulation of PIP_2 production. Given the evidence for the ability of this one lipid to participate in so many intracellular processes, it is increasingly likely that PIP_2 can act as a signalling molecule in its own right. The apparent ability of various G proteins to regulate its synthesis in distinct intracellular compartments is very much in keeping with the idea that functionally distinct, independent PIP_2 pools do indeed occur within cells.

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